Occurrence of integrons and resistance genes among sulphonamide-resistant *Shigella* spp. from Brazil

Gisele Peirano¹, Yvonne Agersø², Frank M. Aarestrup²* and Dalia dos Prazeres Rodrigues¹

¹Oswaldo Cruz Institute, Avenida Brasil 4365, 21045–900 Rio de Janeiro, Brazil; ²Danish Institute for Food and Veterinary Research, Bülowsvej 27, DK-1790 Copenhagen V, Denmark

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**Objectives:** To determine the occurrence of class 1 and 2 integrons and antimicrobial resistance genes among sulphonamide-resistant *Shigella* strains isolated in Brazil during 1999–2003.

**Methods:** Sixty-two *Shigella* (*Shigella flexneri*, *n* = 47 and *Shigella sonnei*, *n* = 15) were tested against 21 antimicrobial agents. The presence of integrons classes 1 and 2 and antimicrobial resistance genes was investigated by PCR using specific primers.

**Results:** A total of eight antimicrobial resistance profiles were identified, with the profile of resistance to sulfamethoxazole, trimethoprim, spectinomycin, streptomycin and tetracycline being the most common among *S. sonnei*, and additionally to ampicillin and chloramphenicol among *S. flexneri*. Class 1 integrons were found in only two strains, whereas class 2 integrons were found in 56 (90.3%) of the strains. All class 2-positive strains had a similar fragment of 2214 bp harbouring a gene cassette array conferring resistance to trimethoprim, streptothricin and spectinomycin/streptomycin. The genes coding for resistance to chloramphenicol (*catA1*), tetracycline [*tet*(A) and *tet*(B)] and ampicillin (*bla*OXA and *bla*TEM), were detected in resistant strains.

**Conclusions:** The detection of class 1 and 2 integrons and additional antimicrobial resistance genes allowed us to identify the most frequent antimicrobial resistance patterns of *Shigella* spp. isolated in Brazil.

**Keywords:** *Shigella sonnei*, *Shigella flexneri*, tetracycline, chloramphenicol, OXA and TEM β-lactamases

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**Introduction**

Shigellosis is endemic throughout the world. Worldwide there are ~165 million cases, of which 163 million are in developing countries and 1.5 million in industrialized countries.¹ Each year 1.1 million people are estimated to die from *Shigella* infection and nearly 580,000 cases of shigellosis are reported among travellers from industrialized countries. A total of 69% of all episodes and 61% of all deaths attributable to shigellosis involve children <5 years of age.¹ *Shigella dysenteriae* and *Shigella flexneri* are the predominant species in tropical areas, whereas *Shigella sonnei* is predominantly isolated in industrialized countries. Besides the self-limiting duration of the disease, effective antimicrobial therapy reduces the duration and severity of the dysentery and can also prevent potentially lethal complications.

Concomitantly, the excretion of the pathogen in stools is shortened significantly, reducing spread of the infection.² Antimicrobial resistance in enteric pathogens is of major concern in developing countries, where the rates of diarrhoeal diseases are highest due to socioeconomic and behavioural factors. Because of the increased resistance to most of the widely used and inexpensive antibiotics (ampicillin, nalidixic acid, co-trimoxazole, tetracycline and chloramphenicol), effective treatment is becoming increasingly difficult.³,⁴

The role of integrons and gene cassettes in the dissemination of multidrug resistance in Gram-negative bacteria is well-established,⁵ and they have been frequently reported among members of *Enterobacteriaceae* of both human and animal origins, being associated with horizontal transfer of antimicrobial resistance genes. To date, there are five different integron classes, each...
with a distinct integrase gene, associated with gene cassettes coding for antimicrobial resistance genes. Knowledge on the epidemiology and molecular mechanisms of antimicrobial resistance in this important pathogen is essential to implement intervention strategies. This study was conducted to investigate the occurrence of sulphonamide and additional resistance genes, and class 1 and 2 integrons as mediators of antimicrobial resistance among Shigella spp. isolated from humans in Brazil.

**Materials and methods**

**Bacterial isolates**

This study comprised 62 Shigella (S. flexneri, n = 47 and S. sonnei n = 15) strains selected from the National Reference Laboratory for Cholera and Enteric Diseases (NRLCED)—IOC/FIOCRUZ, Rio de Janeiro, Brazil. These strains were isolated from faecal samples in Cholera and Enteric Diseases (NRLCED)—IOC/FIOCRUZ, Rio de Janeiro, Brazil. These strains were isolated from faecal samples in Brazilian Regional Laboratories during 1999–2003 and sent to janeiro, Brazil. These strains were isolated from faecal samples in Brazilian Regional Laboratories during 1999–2003 and sent to NRLCED for further identification and serotyping as part of a surveillance programme on antimicrobial resistance. Selection criteria were sulphonamide/sulfamethoxazole resistance. Selected resistant strains were maintained in BHI broth +15% glycerol at –80°C. Further analysis.

**Susceptibility testing**

MIC determinations were performed using a commercially dehydrated panel, Sensititre (Trek Diagnostic Systems, UK), as described by Aarestrup et al. Susceptibility testing was performed by standard disc diffusion on Mueller–Hinton agar plates using antimicrobial discs (Oxoid, UK): cefoxitin (30 μg), cefepime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), imipenem (10 μg) and kanamycin (30 μg). The following quality control strains were used: Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27852), Enterococcus faecalis (ATCC 29212) and Staphylococcus aureus (ATCC 29213). The interpretation of MIC breakpoints and zone diameter inhibition was as recommended by the NCCLS.

**Detection of integrons and resistance genes**

PCR was used to detect the presence of integrons and antimicrobial resistance genes. Bacteria were suspended in water and lysed by boiling as previously described. This study, 12 Class 1 and 2 integron primers and PCR amplifications were designed and performed according to Sandvang et al. and White et al., respectively. Sulphonamide resistance genes sul2 and sul3 were amplified with a specific pair of primers each. Additional resistance genes were investigated in ampicillin-resistant strains for blaOXA and blaTEM genes, chloramphenicol-resistant strains for

**Table 1. Oligonucleotide primer sequences used for amplification of class 1 and 2 integrons and resistance genes**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>qacED1</td>
<td>5′-ATCGCAATAGTGGGCGAAGT-3′</td>
<td>226</td>
<td>11</td>
</tr>
<tr>
<td>qacED1-sul1</td>
<td>5′-ATCGCAATAGTGGGCGAAGT-3′</td>
<td>798</td>
<td>11</td>
</tr>
<tr>
<td>intI1</td>
<td>5′-AAGCAGACT TGACCTGT-3′</td>
<td>variable length</td>
<td>11</td>
</tr>
<tr>
<td>intI2</td>
<td>5′-GCGACACTG CGAAAGC-3′</td>
<td>789</td>
<td>12</td>
</tr>
<tr>
<td>intI2 (variable region)</td>
<td>5′-GATGAGATGACACTG-3′</td>
<td>2214</td>
<td>This study, 12</td>
</tr>
<tr>
<td>sul2</td>
<td>5′-GCAGCTCAAGGCGAATGGCAT-3′</td>
<td>285</td>
<td>7</td>
</tr>
<tr>
<td>sul3</td>
<td>5′-GCAGCTCAAGGCGAATGGCAT-3′</td>
<td>799</td>
<td>34</td>
</tr>
<tr>
<td>blaOXA</td>
<td>5′-ATGAGGGAATACTACTGCG-3′</td>
<td>820</td>
<td>35</td>
</tr>
<tr>
<td>blaTEM</td>
<td>5′-ATGAGATTCATTACATTCG-3′</td>
<td>859</td>
<td>35</td>
</tr>
<tr>
<td>catA1</td>
<td>5′-GTCGTCGTAATGCGGCTACG-3′</td>
<td>457</td>
<td>7</td>
</tr>
<tr>
<td>tet(A)</td>
<td>5′-GTCGTCGTAATGCGGCTACG-3′</td>
<td>957</td>
<td>36</td>
</tr>
<tr>
<td>tet(B)</td>
<td>5′-GTCGTCGTAATGCGGCTACG-3′</td>
<td>415</td>
<td>37</td>
</tr>
<tr>
<td>tet(C)</td>
<td>5′-GTCGTCGTAATGCGGCTACG-3′</td>
<td>506</td>
<td>37</td>
</tr>
<tr>
<td>tet(D)</td>
<td>5′-GTCGTCGTAATGCGGCTACG-3′</td>
<td>436</td>
<td>38</td>
</tr>
</tbody>
</table>

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catA1 gene, and tetracycline-resistant strains for tet(A), tet(B), tet(C) and tet(D) genes. Primer sequences are listed in Table 1.

**DNA sequencing**

PCR products generated by the variable regions of both class 1 and 2 integrons (n = 2 of each class of integron), three *bla*OXA and six *bla*TEM were purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK) according to the manufacturer’s instructions. Sequencing was performed with an ABI 377 automatic sequencer using the PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Resulting sequences were analysed using the computer program VECTOR NTI Suite 8 (InforMax, Inc.) and compared with the sequences in GenBank.

**Results**

All strains were screened for resistance to 21 antimicrobials and the following numbers of strains were resistant to spectinomycin (59), tetracycline (57), ampicillin (49), chloramphenicol (42) and cefalothin (1) (Table 2). Thirty strains showed intermediate susceptibility (MIC = 16/8 mg/L) to amoxicillin/clavulanic acid, and all were resistant to sulfamethoxazole, streptomycin and trimethoprim. All strains were fully susceptible to nalidixic acid, ciprofloxacin and gentamicin. All but one (cefalothin resistant) was cephalosporin-susceptible. A total of eight antimicrobial resistance profiles were identified. AMP, SMX, TMP, SPT, STR, TET, CHL (n = 38) were the most common among *S. flexneri* and SMX, TMP, SPT, STR, TET (n = 12) among *S. sonnei*.

*sul1* gene was detected, as part of a class 1 integron, in only two strains; however, *sul2* was detected in all 62 strains, including those that were class 1-integron positive. All strains were *sul3* negative. Class 1 integrons were found in *S. flexneri* (1) and *S. sonnei* (1), whereas class 2 integrons were found in a total of 56 strains, including those that were class 1-integron positive. All strains were *sul2* negative.

Class 1 integrons were found in *S. flexneri* (1) and *S. sonnei* (1), whereas class 2 integrons were found in a total of 56 strains, including those that were class 1-integron positive. All strains were *sul2* negative. Class 1 integrons were found in *S. flexneri* (1) and *S. sonnei* (1), whereas class 2 integrons were found in a total of 56 strains, including those that were class 1-integron positive. All strains were *sul2* negative.

**Table 2.** Number of *Shigella* strains resistant to antimicrobials

<table>
<thead>
<tr>
<th></th>
<th>No. tested</th>
<th>AMP</th>
<th>AMX/CLA</th>
<th>CEP</th>
<th>CHL</th>
<th>SPT</th>
<th>STR</th>
<th>SMX</th>
<th>TET</th>
<th>TMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em></td>
<td>47</td>
<td>46</td>
<td>30</td>
<td>1</td>
<td>41</td>
<td>46</td>
<td>47</td>
<td>47</td>
<td>44</td>
<td>47</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>15</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>13</td>
<td>15</td>
<td>15</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>49</td>
<td>30</td>
<td>1</td>
<td>42</td>
<td>59</td>
<td>62</td>
<td>62</td>
<td>57</td>
<td>62</td>
</tr>
</tbody>
</table>

*Based on MIC test: AMP (ampicillin), AMX/CLA (amoxicillin/clavulanic acid), CEP (cefalothin), CHL (chloramphenicol), SPT (spectinomycin), STR (streptomycin), SMX (sulfamethoxazole), TET (tetracycline), and TMP (trimethoprim).

**Table 3.** Distribution of integrons and antimicrobial resistance genes among *Shigella* spp.

<table>
<thead>
<tr>
<th></th>
<th>sul1</th>
<th>sul2</th>
<th>int1</th>
<th>int12</th>
<th><em>bla</em>OXA</th>
<th><em>bla</em>TEM</th>
<th>catA1</th>
<th>tet(A)</th>
<th>tet(B)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em></td>
<td>1</td>
<td>47</td>
<td>1</td>
<td>1</td>
<td>44b</td>
<td>43</td>
<td>3</td>
<td>41</td>
<td>1c</td>
</tr>
<tr>
<td><em>S. sonnei</em></td>
<td>1</td>
<td>15</td>
<td>1</td>
<td>13</td>
<td>–</td>
<td>3</td>
<td>1</td>
<td>1c</td>
<td>12</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td>62</td>
<td>2</td>
<td>57</td>
<td>43</td>
<td>6</td>
<td>42</td>
<td>2</td>
<td>55</td>
</tr>
</tbody>
</table>

*a*Detected as part of class 1 integron.

*b*Including one isolate positive for both classes of integrons.

*c*tet(A) + tet(B).

No isolate was positive for *sul3*, tet(C), or tet(D).

**Discussion**

Increased incidence of resistance to antimicrobial agents among *Shigella* spp. presents a major threat in the control of shigellosis. Indiscriminate use of drugs and horizontal gene transfer has led to *Shigella* species becoming resistant to commonly used antibiotics. Initially, both sulphonamides and tetracycline were effective in the treatment of shigellosis, but since strains rapidly developed resistance to these agents, ampicillin and trimethoprim—sulfamethoxazole were then used. In many parts of the world, strains of all *Shigella* species have become resistant to all these low-cost antimicrobials, and quinolones—such as norfloxacin or ciprofloxacin—are one of the few remaining groups of
effective drugs. Several studies have determined the resistance profiles of Shigella. These studies were mainly based on phenotypic antimicrobial susceptibility tests and all of them showed a high prevalence of antimicrobial resistance among Shigella isolates of different geographical regions. Multiple resistant strains have occurred in Europe, Africa, Asia and South America.

In the present study, 62 sulphonamide-resistant Shigella strains isolated in Brazil during 1999–2003 were analysed for the occurrence of integrons and characterization of genes responsible for antimicrobial resistance.

Among the 62 Shigella strains analysed, only two had a class 1 integron that was located on a transferable plasmid. In conjugation experiments, we observed the co-transfer of intI1 and blatTEM (both strains) and additionally of catA1 and tet(B) (one strain). The transfer of intI2 of a class 2 integron plasmid-positive strain was not observed, which suggested that the class 2 integron was not located on a conjugative plasmid (data not shown).

The proportion of class 1-positive isolates was smaller than initially expected, since recent studies usually detected class 1 integrons in clinical isolates of enterobacteria. In a recent study, class 1 integron-harboring gene cassettes (dfrA12, orfF, adaA2 and oxal1) were detected in 13% of Shigella spp. isolates from Vietnam. However, among S. sonnei the presence of class 2 integrons was more frequently detected, and in this study it was confirmed that they could also be transferred to S. flexneri isolates.

The class 1 integron differs from class 2 in that it is able to integrate and excise gene cassettes, and contains a sulphonamide resistance gene (sul1) in the 3′ conserved segment. Class 2 integrons do not contain the sul1 or qacEΔ1 genes, but rather genes that promote the function of Tn7 transposition. Tn7 has a defective integrase gene intl2 located near the cassette. This defective gene is unable to alter the gene cassette array. The promiscuous nature of Tn7 is thought to have contributed to the rapid dissemination of trimethoprim- and spectinomycin/streptomycin-resistant bacteria. The ability of Tn7 to use both site-specific and non-specific modes of transposition could explain why it has become so widespread and persistent in bacterial populations, as has been observed in Shigella spp. dfrA1 is found in Tn7 and has been reported to be prevalent in S. sonnei. The presence of class 2 integrons in almost all Shigella isolates explains the resistance phenotype of streptomycin/pectinomycin and trimethoprim resistance observed in these strains. Streptomycin resistance is strongly associated with integrons because of the high prevalence of adaA cassettes within both class 1 and 2 integrons. Besides the low prevalence of class 1 integrons, the sulphonamide-resistant phenotype could be explained by detection of sul2 genes in almost all strains, independent of the integron class detected. The high frequency of sulphonamide (encoded by sul2) and trimethoprim resistance is probably caused by the frequent use of trimethoprim—sulfamethoxazole for the treatment of shigellosis.

Most of the ampicillin-resistant strains were associated with the presence of an OXA-type β-lactamase (OXA-30). The predominance of OXA-type β-lactamase in Shigella has also been reported previously from other geographical areas. In our study, TEM-1 was detected in only six strains (three S. flexneri and three S. sonnei). TEM-type β-lactamase has previously been detected in conjugally transferable R-plasmids in S. sonnei isolated in Korea and among isolates of S. flexneri in Chile.

The chloramphenicol resistance gene catA1, which codes for chloramphenicol acetyltransferase, was detected in all chloramphenicol-resistant strains, explaining the high level of chloramphenicol resistance due to enzyme activity. This gene has already been detected and studied among S. flexneri isolates in Chile.

Our results showed that tet(B) was the predominant tetracycline-resistant gene in both species. Another recent study also concluded that tet(B) was the predominant tetracycline resistance determinant among all serogroups examined in 532 Shigella strains, especially among S. flexneri.

In summary, all isolates were phenotypically resistant to the antimicrobials encountered by the resistance genes encoded not only within the gene cassettes of class 1 and 2 integrons but also by integron-independent genes.

By using PCR and DNA sequencing techniques we determined the content and order of the antibiotic resistance genes inserted between the conserved segments of two classes of integrons and additionally identified the main resistance genes involved in antimicrobial patterns of resistant Shigella isolates. In addition, molecular techniques, such as PCR assays, could be valuable tools for resistance mechanism studies. They provide epidemiological information about the occurrence and transmission of resistance genes through bacterial populations and the geographical and temporal spread of particular clones, allowing discrimination between clonal spread and horizontal transfer of resistance determinants.

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References

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